Folate-mediated chemotherapy and diagnostics: an updated review and outlook

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Abstract

Folate receptor (FR) is highly expressed in many types of human cancers compared to normal cells, and it has been actively studied for developing targeted chemotherapy. Tremendous efforts have been made in developing FR-targeted nanomedicines and translating them into clinical applications. This article provides a concise review on the latest development of folate-mediated nanomedicines and nanoprobe for chemotherapy and diagnostics with an emphasis on in vivo applications. The cellular uptake mechanisms, pharmacokinetics (PK), administration routes and major challenges in FR-targeted nanoparticles are discussed.

Keywords: Folic acid; folate receptor; targeted delivery; nanomedicine; diagnosis

1. Introduction

Folate, also known as vitamin B₉, naturally occurs in food, and folic acid (FA) is a synthetic form of this vitamin. Folate plays an essential role in one-carbon transfer reactions, which are involved in DNA synthesis and replication, cell division, growth, and survival, particularly in rapidly dividing cells. It is also required for biosynthesis of S-adenosylmethione, which is essential for methylation of DNA, RNA, proteins, and phospholipids [1]. In adult tissues, folate or FA is mainly transported by two transporters: reduced folate carrier (RFC) and folate receptors (FRs). RFC, a ubiquitously expressed anion channel, is a low affinity ($K_m = 1-10 \mu\text{M}$) but high capacity transporter [2, 3]. In contrast, FRs, mainly found in kidney, lung, and placenta, are high affinity but low capacity transporters. Three subtypes of FRs have been identified: FR α , FR β , and FR γ . Among them, FR α is most widely expressed at very low levels in normal tissues, but it is highly expressed in numerous cancers including ovarian, pediatric ependymal brain, mesothelioma, breast, colon, renal, lung tumors, and head and neck carcinomas, in order to meet high folate demand of rapidly dividing cells under low folate conditions [4].

FR α , a membrane-associated form of FR, is normally expressed at the apical (luminal) surface of epithelial cells to keep itself away from and out of direct contact with folate in the circulation [5, 6]. However, carcinoma cells allow FR α accessible to folate in the circulation by alteration of the transmembrane region, transcriptional level and translational level of FR α [7-9]. Elevated FR α expression could increase folate uptake to stimulate cellular DNA repair during the

early stages of carcinogenesis. The continued expression of FR α could then support the transition of cellular environment that favors uncontrolled rapid growth of tumor cells [10]. The FR α -positive tumor (nonmucinous epithelial ovarian carcinoma) reveals that the level of FR α protein expression is closely associated with tumor progression [11]. Because of high tumor-specificity, the exploitation of FR-targeted nanomedicines and nanoprobe rapidly advances cancer chemotherapy and diagnostics. As a potential targeting moiety, FA holds several advantages including low molecular weight and immunogenicity, relatively high stability, and ease of synthesis, compared with most antibodies [12].

Tremendous efforts have been made in developing FR-targeted nanomedicines and translating them into clinical applications. A few FR-targeted chemotherapies have entered clinical trials for cancer treatment. EC0489, a FR-targeted drug conjugate consisting a vinca alkaloid microtubule destabilizing agent linked to an FA molecule, and EC0225, a FR-targeted two drugs conjugate containing two potent cytotoxic agents of vinca alkaloid and mitomycin C linked to a single FA molecule, are under development by Endocyte Inc [13]. BMS753493, an antimitotic agent containing an epothilone moiety linked to a single FA molecule, is under development by Endocyte Inc. and Bristol Myers Squibb [14]. Vintafolide, a small molecular drug conjugate consisting a potent chemotherapeutic drug of vinblastine linked to an FA molecule, is under development by Endocyte Inc. and Merck & Co [15]. Mirvetuximab soravtansine (IMGN853), an antimitotic agent (maytansinoid DM4) conjugated to an

FR α -targeting antibody (M9346A), is under development by ImmunoGen Inc. A vaccine adjuvant, FR α peptide vaccine mixed with granulocyte macrophage colony-stimulating factor (GM-CSF), is currently under development by TapImmune Inc. In addition to the ongoing clinical trials, numerous FR-targeted chemotherapies have been developed in the laboratories worldwide. These chemotherapies can be divided by different categories including FA-drug conjugates, FR-targeted nanoparticles, FR-targeted immunotherapies, and FR-targeted imaging and diagnosis. Early findings of folate-related chemo- or immune-therapies have been reviewed and discussed in the twentieth century [9, 16-20]. This article provides a concise review on latest advances in FR-targeted nanomedicines with an emphasis on in vivo findings.

2. Structural basis of folic acid and folate receptors

FA is composed of three sub-components, i.e., pterin, para-amino-benzoic acid, and glutamic acid (**Figure 1**). A recently molecular simulation and docking study revealed the binding sites of FA and FR α [4]. In this study, FA was docked into an extended groove of FR α in the direction roughly perpendicular to the plane formed by helices $\alpha 1$, $\alpha 2$ and $\alpha 3$. Both hydrogen bonds and hydrophobic interactions occurred around the pteridine ring of FA. The ligand-binding affinity study showed that -NH₂^a of FA bound to D81A of FR α . Mutation of D81 of FR α greatly increased the dissociation constant (K_d) value by more than one order of magnitude, the largest increase observed in the study, suggesting the interaction of the aspartate carboxyl oxygens with the pterin N1 and N2 nitrogens of FA is very strong and contributes to the highest binding

affinity between FA and FR α . In contrast, -OH^b of FA bound to R103A, R106A and S174A of FR α ; -COOH^c of FA bound to W140A of FR α ; and -COOH^d of FA bound to W102A and K136A of FR α . Mutations of K136 and R106 have little effect, and mutations of W102, R103, W140, and S174 have moderate effects on folic acid binding [4]. This study illustrates the key binding site of FA to bind to FR α , which provides a structural requirement of the pterin group, especially -NH₂^a function group, for anchoring folate in the binding pocket of the receptor. Given that, the glutamate group of FA, especially -COOH^{c,d}, can be used for conjugation with linkages/spacers, drug, contrast reagents or nanoparticles, without significantly compromising the interactions between receptor and ligand.

Figure 1. Structure of folic acid

3. FA-decorated nanomedicines

Most FA cannot dissolve in water because of its low solubility in water (0.0016 mg/mL at 25 °C). Hence, FA is commonly chemically conjugated onto water soluble polymers or

integrated onto the nanoparticles to prolong the circulation time and guide drug or nanoparticles to the FR highly expressed tumors [12]. Polyethylene glycol (PEG) modification is generally known to increase the solubility of the drug and prolong the half-life of drug in the body, which may passively increase drug accumulation at tumor due to the enhanced permeation and retention (EPR) effect. Additional FA modification may increase retention of the nanoparticle in the tumor mass and facilitate uptake of the nanoparticle by FR-mediated endocytosis, which enables the drug to arrive the FR-highly expressing tumor cells more efficiently and precisely [21]. FA-decorated nanoparticles can be generally synthesized as shown in **Figure 2** with minor modification depending on the unique properties of various nanoparticles. A variety of FR-targeted nanomedicines have been developed based on polymer-drug conjugates, liposomes, polymeric micelles, dendrimers, and other nanoparticles such as nanotubes, nanosheets, etc. Most studies suggest that FA decoration allows nanoparticles to enhance drug and gene delivery efficiency to the tumor site, and the major recent in vivo findings were summarized in **Table 1**.

Figure 2. Synthetic scheme of FA-mediated nanoparticle (FA-NP)

Table 1. Major recent pre-clinical findings of FR-targeted nanomedicines

Nanocarrier	Drug	Model	Route	Major findings	Ref.
FR-targeted (FRT) liposome	Carboplatin	IGROV-1 ovarian tumor-bearing SCID BC-17 mice	i.p.	<ul style="list-style-type: none"> ● A superior survival rate (5 out of 6 of mice) was observed in the mice treated with FRT carboplatin liposome twice a week for 3 weeks, and no metastasis was observed in these mice. In contrast, no survivor was observed in the mice treated with saline, free carboplatin or non-FR-targeted carboplatin liposome, and the cancer cells metastasized to the lung and liver tissues in these mice. 	[22]
FA and TAT peptide conjugated, octadecyl-quaternized, lysine-modified chitosan-cholesterol polymeric liposomes (FA-TATp-PLs)	Paclitaxel (PTX)	KB nasopharyngeal tumor-bearing SCID mice	i.v.	<ul style="list-style-type: none"> ● The tumor growth rate of the mice treated with PTX-loaded FA-TATp-PLs or free Taxol was reduced by 71% and 49%, respectively, compared with the one of PBS-treated mice. ● The drug delivery system, FA-TATp-PLs, had no effect on tumor growth. 	[23]
FA-coupled PEGylated nano-paclitaxel liposome (FA-NP)	Paclitaxel (PTX)	SKOV3/TAX ovarian tumor-bearing nude mice	i.p. i.v.	<ul style="list-style-type: none"> ● i.p. administration of FA-NP but not free PTX or non-targeted NP significantly prolonged the survival and reduced tumor nodule number. ● i.v. administration of FA-NP failed to achieve these antitumor effects at the same dose of i.p. administration. 	[24]
FR-targeted docetaxel-lipid-based-nanosuspensions (tLNS)	Docetaxel (DTX)	B16 tumor-bearing Kunming mice	i.v.	<ul style="list-style-type: none"> ● An obvious tumor regression was observed in the mice treated with tLNS, non-targeted docetaxel-lipid-based-nanosuspensions (pLNS), and Duopafei. ● The tumor inhibition rate in the tLNS mice was higher than that in pLNS and Duopafei groups. 	[21]

				<ul style="list-style-type: none"> ● Both tLNS and pLNS induced less toxicity to the mice than Duopafei via i.v. administration. ● The overall targeting efficiencies of pLNS and tLNS were 1.09 and 1.23 times better than that of Duopafei, respectively. 	
FA-conjugated and Rhodamine (RHO)-labeled PAMAM dendrimer generation 4 (G4-RHO-FA) emulsified with oleic acid-coated Fe ₃ O ₄ NPs (OA-Fe ₃ O ₄) in the lipid solution to form Mag-pH-DS dendrosomes	DiR dye	HeLa tumor-bearing BALB/c nude mice	i.v.	<ul style="list-style-type: none"> ● Both non-magnetic pH-DS and Mag-pH-DS enhanced targeting efficiency compared with G4-RHO-FA. ● Mag-pH-DS treated animals combined with an external magnetic field had a shorter peak time with a nearly twice the peak intensity compared with that without magnetic field. ● Mag-pH-DS exhibited superior tumor accumulation compared with G4-RHO-FA and pH-DS. ● Dendrosomes had higher peak intensity and a longer residence time than G4-RHO-FA. ● The major elimination routes of both non-pH sensitive DS, pH-DS and Mag-pH-DS were liver and spleen; whereas G4-RHO-FA was primarily eliminated by the kidney. 	[25]
A heparin-folate-paclitaxel (HFT) backbone with an additional paclitaxel (T) loaded in its hydrophobic core (HFT-T)	Paclitaxel (PTX)	KB-3-1 and KB-8-5 (PTX-resistant) tumor-bearing nude mice	i.v.	<ul style="list-style-type: none"> ● HFT-T enhanced the specific delivery of PTX into tumor tissues, prolonged retention of PTX within tumor tissues, and markedly retarded tumor growth. ● The enhanced antitumor effects were associated with a higher degree of microtubule stabilization, mitotic arrest, antiangiogenic activity, and inhibition of cell proliferation. 	[26, 27]

FA-conjugated poly(ester amine) (FP-PEA) complexed with TAM67 gene (FP-PEA/TAM67 polyplexes)	TAM67 gene	KB tumor-bearing BALB/c nude mice	i.t.	<ul style="list-style-type: none"> ● FR-PEA/TAM67 polyplexes inhibited the tumor growth by suppressing cell proliferation in the tumor. 	[28]
siRNA-loaded FA-PEG-chitosan oligosaccharide lactate NPs (siRNA/FA-PEG-COL)	siRNA against HIF-1 α	OVK18#2 ovarian tumor-bearing BALB nude mice	i.v.	<ul style="list-style-type: none"> ● FA-PEG-COL NPs possessed superior compatibility with erythrocytes in terms of degree of aggregation and hemolytic activity. ● FA-PEG-COL NPs showed significant greater tumor accumulation than non-targeted COL NPs. 	[29]
FA-PEG-chitosan-graft-polyethylenimine (FPCP)	GFP plasmid and <i>Pdcd4</i> plasmid	H- <i>ras</i> 12V transgenic mice	i.v.	<ul style="list-style-type: none"> ● FPCP/GFP complexes showed high levels of GFP expression in liver cancer tissues compared with GFP alone and PEI/GFP complexes. ● FPCP/<i>Pdcd4</i> complexes showed a significant decrease in tumor numbers compared with <i>Pdcd4</i> alone but not PEI-<i>Pdcd4</i> complexes. 	[30]
2-hydroxypopyl- β -cyclodextrin (HP- β -CD) and FA cross-linked with low molecular weight polyethyleneimine (FA-HP- β -CD-PEI)	siRNA against VEGF	HeLa tumor-bearing nude mice	i.v.	<ul style="list-style-type: none"> ● Four doses of FA-HP-β-CD-PEI/siVEGF polyplexes reduced tumor growth by decreasing VEGF expression in the tumor, compared to the ones treated with HP-β-CD-PEI/siVEGF polyplexes. 	[31]
FA-decorated ethylenediamine-surface modified fullerene (C60-PEI-FA)	Docetaxel (DTX)	S180 tumor-bearing BALB/c mice	i.v.	<ul style="list-style-type: none"> ● Administration of C60-PEI-FA/DTX every 2 days significantly inhibited tumor growth, compared to untreated, C60-PEI-FA-, free DTX-, C60-PEI/DTX-treated mice 	[32]
Docetaxel encapsulated PLGA-lecithin-PEG core-shell NPs (FT-NP DTX)	Docetaxel (DTX)	KB tumor-bearing nude mice	i.v.	<ul style="list-style-type: none"> ● The radiosensitization efficacy of FT-NP DTX is dependent on the timing of radiotherapy. ● Free DTX, FT-NP DTX and non-targeted NP DTX (NT-NP DTX) led to significant 	[33]

				tumor growth delay when tumors were irradiated, but FT-NP DTX produced the maximum tumor growth delay, which was significantly different from NT-NP DTX.	
FA-modified stealthy PEOz corona micelles (FA-PEOz-PCL)	Doxorubicin (DOX)	KB tumor-bearing BALB/c nude mice	i.v.	<ul style="list-style-type: none"> ● DOX entrapped FA-PEOz-PCL micelles effectively inhibited the tumor growth and reduced the toxicity to mice compared with free DOX. 	[34]
FA and paclitaxel loaded polymeric micelles [FA-M(PTX)] prepared by co-assembling FA-polymer conjugate [MPEG-b-P(LA-co-DHP/FA)] and PTX-polymer conjugate [MPEG-b-P(LA-co-MCC/PTX)]	Paclitaxel (PTX)	EC9706 esophageal tumor-bearing nude mice	i.v.	<ul style="list-style-type: none"> ● FA-M(PTX) micelles efficiently inhibited tumor growth and extended the survival rate of the mice, compared to free PTX and non-targeted M(PTX) micelles. 	[35]
Folate-decorated biodegradable polymeric micelles [FA-M(Pt)] prepared by co-assembling FA-polymer conjugate (FA-PEG-PLA) and diaminocyclohexane platinum (DACH-Pt) of oxaliplatin-polymer complex [mPEG-b-P(LA-co-MCC/Pt)]	Oxaliplatin	H22 liver tumor-bearing Kunming mice	i.v.	<ul style="list-style-type: none"> ● FA-M(Pt) effectively inhibited the tumor growth and prolonged the survival rate of the mice, compared to non-targeted M(Pt) and free oxaliplatin. 	[36]
FA-conjugated carboxymethyl lauryl chitosan/superparamagnetic iron oxide (FA-CLC/SPIO) micelles	Chlorin e6 (Ce6)	Hela tumor-bearing nude mice	i.v.	<ul style="list-style-type: none"> ● Targeted sensitization-enhanced radiotherapy (TSER: Ce6-loaded FA-CLC/SPIO micelles and US/XR treatment) greatly inhibited tumor growth; whereas free Ce6 and US/XR, alone or together, did not substantially inhibited tumor growth. ● TSER markedly decreased level of nuclear integrity in the tumor. ● No abnormalities (i.e., within normal ranges of ALT, AST, BUN and CREA) were observed in liver or kidney function in the TSER-treated mice. 	[37]
Poly(propylene sulfide)-PEG-serine-folic acid	Zinc	HepG2 tumor-bearing	i.v.	<ul style="list-style-type: none"> ● PPS-mPEG-Ser-FA@DOX@ZNPC 	[38]

(PPS-mPEG-Ser-FA) emulsified micelles	phthalocyanine (ZNPC) and doxorubicin (DOX)	nude mice		<p>micelles with irradiation induced the greatest tumor suppression.</p> <ul style="list-style-type: none"> ● Laser irradiation enhanced tumor accumulation of DOX, disassociation of micelles, and release of DOX. ● PPS-mPEG-Ser-FA micelles significantly reduce the toxic side effect of DOX for the treatment. 	
Docetaxel and cisplatin-encapsulated liquid crystalline NPs formed by hydroxypropyl- β -cyclodextrin mixed with chitosan-PEG-folic acid (FA-LCN/DC)	Docetaxel (DOC) and cisplatin (CIS)	MDA-MB-231 tumor-bearing Balb/c nude mice	i.v.	<ul style="list-style-type: none"> ● The inhibition of tumor growth was greater in mice treated with FA-LCN/DC than those with DOC, CIS, and DC cocktail. ● The survival rate of mice treated with FA-LCN/DC was higher than those with control, DOC, CIS, and DC cocktail. ● The tumor caspase-3 and RARP expression levels were elevated and Ki-67 and CD31 were reduced in the mice treated with FA-LCN/DC than those with DOC, CIS, and DC cocktail. 	[39]
FA-PEG-PCL and MPEG-PCL emulsified NPs (FANPs)	DiR dye	A549 tumore-bearing nude mice	i.v.	<ul style="list-style-type: none"> ● The tumor accumulation of FANP in DTX treated tumor was higher than untreated tumor on the same mouse. ● Pretreatment with DTX largely enhanced the accumulation of NP and FANP in tumor sites, and the fluorescent intensity in FANP group was 1.2 fold higher than NP group. ● The fluorescence mainly focused in liver and spleen. 	[40]
FA and 1,2-dicarboxylic-cyclohexene anhydride (DCA)-functionalized n-butylamine-poly(L-lysine)-b-poly(L-cysteine) (FA-PLL(DCA)-PLC) NPs (charge-reversible)	Doxorubicin (DOX)	Hela tumor-bearing nude mice	i.v.	<ul style="list-style-type: none"> ● The amount of DOX accumulated in tumors treated with DOX-loaded FD-NPs was much higher than that with free DOX and DOX-loaded FS-NPs. 	[41]

FD-NPs) and negatively charged FA-PLL(succinic anhydride)-PLC NPs (FS-NPs)					
FA-decorated self-organized NPs fabricated by complexation of doxorubicin-conjugated dextran with FA-grafted chitosan (FADex NPs)	Doxorubicin (DOX)	KB tumor-bearing BALB/c nude mice	i.v.	<ul style="list-style-type: none"> ● Two doses of FADex NPs efficiently suppressed the tumor growth, compared to non-targeted PEGylated NPs (CPDex NPs) and free DOX. ● This tumor growth suppression was significantly prohibited in the presence of free FA (2 mM), as the tumor growth tendency of FADex NPs and FA-treated mice became similar to the one of CPDex NPs-treated mice. 	[42]
pH-sensitive FA-PEG-chitosan-PAMAM-plasmid DNA polyplexes (FPCPHDs)	RFP plasmid	S180 tumor-bearing BALB/c nude mice	i.v.	<ul style="list-style-type: none"> ● FPCPHDs significantly increased RFP expression at the tumor site, compared to non-targeted PDs, PHDs, CPHDs, and PCPHDs polyplexes. 	[43]
Self-assembled polyelectrolyte polyplexes of folate-dextran-siRNA via disulfide bonds and linear polyethylenimine (folate-DSC/LPEI/siRNA)	siRNA against GFP (siGFP)	GFP overexpressing-HeLa tumor-bearing nude mice	i.v.	<ul style="list-style-type: none"> ● Folate-DSC/LPEI/siGFP efficiently suppressed GFP expression in tumor, compared to PBS and non-targeted DSC/LPEI/siGFP. 	[44]
A molecular hydrogelator system of FA-Taxol conjugates formed by glutathione	Taxol	4T1-luciferase breast tumor-bearing mice	i.t.	<ul style="list-style-type: none"> ● A single dose of i.t. injection of FA-Taxol hydrogel more efficiently inhibited tumor growth than four doses of i.v. injection of free Taxol. 	[45]
A FR-targeted rhaponticin (FRHA) synthesized via a releasable disulfide linker	Rhaponticin (RHA)	FR-positive KB and M109 tumor-bearing Balb/c mice FR-negative 4T1 tumor-bearing Balb/c mice	i.v.	<ul style="list-style-type: none"> ● Administration of FRHA to KB and M109 tumor-bearing mice three times per week significantly inhibited tumor growth, compared to PBS and free RHA treatment. ● Administration of FRHA to 4T1 tumor-bearing mice failed to suppress tumor growth. 	[46]
Folate-appended methyl- β -cyclodextrin	FA-M- β -CyD	Colon-26	i.t.	<ul style="list-style-type: none"> ● FA-M-β-CyD drastically inhibited the tumor 	[47]

(FA-M- β -CyD)		tumor-bearing BALB/c mice	i.v.	<p>growth, compared to control, doxorubicin, and non-targeted M-β-CyD.</p> <ul style="list-style-type: none"> ● All of the tumor-bearing mice treated with FA-M-β-CyD survived for at least 140 days; whereas the others only survived for around 60 days. ● FA-M-β-CyD treated mice showed no significant change in blood chemistry values, including creatinine, blood urea nitrogen, aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase. 	
FA-anchored cucumber mosaic virus (FA-CMV)	Doxorubicin (DOX)	OVCAR-3 tumor-bearing BALB/c nude mice	i.p.	<ul style="list-style-type: none"> ● Administration of FA-CMV-DOX every five days significantly decreased the accumulation of DOX in mouse myocardial cells but increased the uptake of DOX in the ovarian cancer cells in tumor tissue, compared to negative control, free DOX, and CMV-DOX treatment. ● FA-CMV-DOX induced less cardiotoxicity and enhanced antitumor effect. 	[48]
FA-conjugated single-walled carbon nanotubes complexed with docosanol-conjugated paclitaxel (FA-SWNT-lipid-PTX)	Paclitaxel (PTX)	MCF-1 breast tumor-bearing nude mice	i.v.	<ul style="list-style-type: none"> ● Four doses of FA-SWNT-lipid-PTX significantly suppressed tumor growth, compared to free PTX and PBS control. ● No mortality and adverse effect was observed in SWNT-lipid-PTX-treated mice. ● Hematologic assessment (hematocrit, hemoglobin, red blood cell count and white blood cell count) and histological staining (liver, heart, lung, and kidney) showed no difference between control and SWNT-lipid-PTX-treated mice. 	[49]

Folate-decorated poly(ethylene oxide)- <i>b</i> -poly(methacrylic acid)-cross-linked nanogels (FA-nanogels)	Cisplatin (CDDP) Doxorubicin (DOX)	A2780 ovarian tumor-bearing nude mice	i.v.	<ul style="list-style-type: none"> ● Tumor uptake of FA-nanogels was significantly higher than free CDDP and non-targeted nanogels at 4 days post-injection. ● The increased uptake was significantly reversed by co-administration of free FA. ● Administration of FA-nanogel/CDDP every four days significantly reduced tumor growth, compared to dextrose, nanogel alone, free CDDP, non-targeted nanogel/CDDP, and FA-nanogel/CDDP with free FA treatment. 	[50]
Doxorubicin-loaded folate-decorated silica nanorattles (DOX-FA-SNs)	Doxorubicin (DOX)	HeLa tumor-bearing BALB/c nude mice	i.v.	<ul style="list-style-type: none"> ● Near infrared-labeled FA-SNs (ICG-FA-SNs) were distributed into the tumor at 4 hours post-injection and retained in the tumor up to 24 hours. ● Administration of DOX-FA-SNs every three days significantly suppressed tumor growth, compared to control, FA-SNs alone, free DOX, and non-targeted DOX-SNs 	[51]

Abbreviations: SCID, severe combined immunodeficiency; NPs, nanoparticles; FA, folic acid; FR, folate receptor; PEG, polyethylene glycol; GFP, green fluorescence protein; RFP, red fluorescence protein; i.v., intravenous; i.t., intratumoral; i.p., intraperitoneal.

To understand the mechanism of cellular uptake and the specificity to the FR, investigators challenged FA-conjugated nanoparticles with free FA in FR highly or negatively expressed cancer cell lines. For example, FA-coupled PEGylated nano-paclitaxel liposome (FA-NP) was developed to reverse drug resistance in paclitaxel-resistant (SKOV3/TAX) ovarian cancer [24]. FA-NP markedly inhibited the growth of ovarian cancer cells and caused more G₂/M cell cycle arrest and apoptotic changes in ovarian cancer cells than NP or free paclitaxel. However, these effects were blunted in the presence of free FA (1 mM), which competitively inhibited the receptor-mediated uptake of FA-NP. A separate study also revealed that the presence of FA within the cholesterol domain could enhance liposome-based transfection in cultured KB cells [52]. Zeng et al. developed FA-decorated poly(ethylene oxide)-*b*-poly(methacrylic acid)-cross-linked nanogels (FA-nanogels) to load cisplatin or doxorubicin. The cellular uptake of FA-nanogels in human ovarian carcinomas FR-positive A2780 cells greatly exceeded that of the non-targeted nanogels. However, the cellular uptake of FA-nanogels only reached about 50% of the initial in the presence of 0.5 mM FA and was further suppressed to 20-25% of the initial in the presence of 10 mM FA [50]. Similarly, it was shown that pre-treatment or the presence of free FA (1-2 mM) significantly reduced the cellular uptake or the cytotoxicity potency of FA-conjugated nanomedicines including FA-appended methyl- β -cyclodextrin (FA-M- β -CyD), FA-anchored cucumber mosaic virus (FA-CMV), or FA-decorated self-organized NPs (FADex NPs) [42, 47, 48].

In addition to chemotherapeutic drug, FA-conjugated nanoparticles, particularly

FA-modified dendrimers, have been explored for targeted gene delivery to FR highly expressed tumors. For example, a pH-sensitive FA-PEG-chitosan-PAMAM-plasmid DNA complexes (FPCHDs) were designed for cancer cell targeting gene delivery [43]. FPCPHDs were prepared by a pH-sensitive core-shell system, which contains PEG tethered carboxylated chitosan modified FA, PAMAM dendrimer generation 4, high mobility group box 1 (HMGB1), and plasmid DNA. FPCPHDs were shown to significantly enhance luciferase activity and red fluorescence protein (RFP) expression in KB cells, compared to non-targeted nanocomplexes. However, such enhancement could be significantly inhibited by pre-treatment of free FA (1 mM). Intracellular trafficking of FPCPHDs in KB cells showed that FPCPHDs rapidly escaped from endo-lysosomes and exclusively located in the nucleus at 3 h post transfection. Similarly, the presence of free FA was also shown to drastically decrease the transfection efficiency of FA-conjugated polyplexes, such as FA-conjugated poly(ester amine) (FP-PEA)/plasmid polyplexes and folate-dextran-siRNA/linear polyethylenimine (folate-DSC/LPEI) polyplexes [28, 44].

In our recent study, we found FA-decorated PAMAM dendrimer generation 4 (G4-FA) facilitated the cellular uptake of dendrimers and dendrimer/plasmid polyplexes in a FR-dependent manner. Free FA dose-dependently suppressed the cellular uptake of G4-FA for the same binding site, that is, FR, on the surface of head and neck cancer cells. Furthermore, we found G4-FA was preferentially taken up by HN12 (FR-high) but not U87 (FR-low) cells in a co-culture model. As a result, G4-FA showed a higher cytocompatibility and transfection

efficiency than non-targeted dendrimer in FR-high cells such as HN12 and T98 [53].

The binding constant of the free folate with FR has been reported as 0.01-1 nM [54, 55]. The reported concentration for free FA to compete with FA-conjugated nanoparticles were 1 mM or more, suggesting that FA-conjugated nanoparticles possessed higher binding affinities to cellular FR than free FA. Such encouraging property may be beneficial for nanoparticles to compete free folate for FR targeting in vivo. On the other hand, increasing FA moieties may not further improve cellular uptake of nanoparticles [56]. For example, an optimal amount of FA moieties (0.1-0.2 μmol FA/mg polymer) was reported, above which the uptake of FA-nanogels was gradually decreased [50]. Similarly, we found excess amount of G4-FA conjugates could suppress the cellular uptake of conjugates themselves, most likely due to the saturation of FR on the cell surface [53]. The fundamental understanding of FA-conjugated nanoparticles as targeting vectors can help investigators to better design drug delivery systems for the cancers with high FR expression.

4. Pharmacokinetics (PK) of FA-decorated NPs

Pharmacokinetics (PK) and pharmacodynamics (PD) of drug is routinely investigated in early clinical studies to determine the absorption, distribution, metabolism and excretion of the drug. The PK study of targeted nanomedicines may be also relevant to determine drug concentration within a target tissue such as tumor [57]. A disulfide FR-targeted rhaponticin (FRHA) was synthesized via tethering the thiol-activated FA-spacer to thiol-reactive derivative of

rhaponticin (RHA) to improve anticancer therapeutic efficacy of RHA [46]. The mouse PK profiles of RHA showed that FRHA was rapidly removed from systemic circulation after its i.v. administration to M109 (FR-positive) tumor-bearing mice (half-life about 10 min) but much slower to those 4T1 (FR-negative) tumor bearing mice and normal mice (half-life over 150 min), suggesting FRHA was rapidly taken up by FR-positive tumor following i.v. administration.

FR-targeted docetaxel (DTX)-lipid-based-nanosuspensions (tLNS) were proposed for active-targeted cancer therapy [21]. The PK profiles of DTX in a B16 tumor-bearing mouse model showed that the DTX serum concentration remained measurable at 12 hours-post i.v. administration of tLNS and non-targeted docetaxel-lipid-based-nanosuspensions (pLNS) but not free Duopafei group. Compared to the Duopafei administration, tLNS and pLNS significantly increased the area under the plasma concentration-time (AUC) by 1.59 and 1.66 times, respectively; prolonged the mean residence time (MRT) by 2.40 and 2.41 times, respectively; and decreased the plasma clearance rate (CL). The overall targeting efficiency of pLNS was 1.09 times better than that of Duopafei; whereas the targeting efficiency of tLNS was 1.13 times better than that of pLNS.

FA-decorated biodegradable polymeric micelles [FA-M(Pt)] were prepared by coassembling FA-polymer conjugate (FA-PEG-PLA) and diaminocyclohexane platinum (DACH-Pt) of oxaliplatin-polymer complex [mPEG-b-P(LA-co-MCC/Pt)] for cancer therapy [36]. The PK profiles of Pt in a KB tumor-bearing mouse model showed that FA-M(Pt) possessed greater steady-state AUC, slower CL, and longer MRT than free oxaliplatin. M(Pt) had higher plasma Pt

concentration within 4 h; whereas FA-M(Pt) had higher plasma concentration after 4 h. Compared to M(Pt), FA-M(Pt) showed a bigger AUC, slower CL, but same MRT. FA-M(Pt) was not as good as M(Pt) in respect of the plasma drug concentration, presumably due to the enhanced interaction between the FA moieties on the micelles and the blood proteins compared to the case of M(Pt) which were only PEGylated to prolong the circulation time.

FA-decorated ethylenediamine-surface modified fullerene (C60-PEI-FA) was synthesized for tumor-targeted delivery of docetaxel (DTX) [32]. The PK profiles of DTX in an S180 tumor mouse model showed that C60-PEI-FA/DTX significantly increased the blood circulation time of DTX with an increase of AUC and MRT by 2 and 6 times, respectively, compared to free DTX. Meanwhile, the uptake of DTX in tumor was significantly higher in C60-PEI-FA/DTX-treated mice than in C60-PEI/DTX and DTX-treated mice. All these findings provide strong evidence in rational design of FR-targeted nanomedicines based upon the improved PK profiles.

5. Administration routes of FR-targeted NPs

The administration routes commonly used to get therapeutic and imaging agents to the tumor include intraperitoneal (i.p.) injection, intratumoral (i.t.) injection, intravenous (i.v.) injection, enteric administration, inhaled administration, etc. A majority of FA-drug conjugates and FR-targeted nanomedicines and imaging nanoprobe are systemically administered via i.v. injection as the ease of application and avoidance of the first pass effect. Following systemic administration, FR-targeted NPs can follow enhanced permeability and retention (EPR) effect

and FR-mediated endocytosis to get into the tumor. Compared to the systemic administration, local administration such as i.t. injection or convection-enhanced delivery (CED) is more efficacious and precise to deliver therapeutics to the tumor and reduce adverse-effect [58]. FR-targeted nanomedicines can prolong tumor retention time within the tumor microenvironment. However, such local administration is limited by the tumor origin. Inhaled and enteric drug administrations of FR-targeted nanomedicines have been investigated recently as alternative non-invasive administration routes. For example, FA-PEG-hydrophobically-modified dextran (F-PEG-HMD) micelles were developed for FR-expressing lung tumor drug delivery via pulmonary administration [59]. Spray-drying was applied to obtain dry powders embedding the paclitaxel-loaded F-PEG-HMD micelles. In the M109-HiFR murine orthotopic lung tumor mouse model, F-PEG-HMD micelles reached the lung tumor site and consequently distributed into the lung tumor with no sign of lung toxicity and inflammation after pulmonary administration.

As another example, mesoporous silica NPs (MSNs) functionalized with PEG, PEI, and FA were developed for colon cancer and inflammation drug delivery via enteric administration [60]. In an 18 FVB/N mouse model, the number of intestinal goblet cell differentiation in the colon of mice given γ -secretase inhibitor (DAPT)/FA-PEG-PEI-MSNs were significantly higher than DAPT alone, DAPT/FA-PEI-MSNs, and FA-PEG-PEI-MSNs after gavage. This formulation offers an alternate route for the long-term non-invasive management of intestinal diseases such as colon cancer and inflammation.

6. FR-targeted NPs for tumor diagnostics and theranostics

Nanotechnology has been extensively applied to improve tumor imaging and diagnostics, including near-infrared (NIR) fluorescence imaging, magnetic resonance imaging (MRI), computed tomography (CT), ultrasonic imaging. NIR fluorescence imaging becomes a highly sensitive, non-invasive, non-radiant technique for real-time in vivo monitoring of biological information [61]. In addition to therapeutic agent delivery, FA has been widely used for the selective delivery of attached imaging agents. Major recent findings using FR-targeted nanoprobes for in vivo tumor imaging were summarized in **Table 2**. By combination, an MRI guided photothermal-chemotherapy was developed as a theranostic nanoplatform [62]. Gadolinium (Gd) NPs were decorated onto grapheme oxide (GO) to form GO@Gd nanocomposites, which were then functioned by PEG and FA and loaded with DOX to form GO@Gd-PEG-FA/DOX NPs. In an S180 tumor-bearing mouse model, GO@Gd-PEG-FA/DOX greatly enhanced the uptake of DOX in tumor compared to GO@Gd-PEG/DPX and free DOX at all the time after i.v. administration. GO@Gd-PEG-FA exhibited a good tumor-targeting property and a strong T_1 -weighted MRI ability. Under the guidance with MRI imaging, synergistic treatment of GO@Gd-PEG-FA/DOX with NIR irradiation significantly enhanced the antitumor effect compared to free DOX, GO@Gd-PEG/DOX, GO@Gd-PEG-FA/DOX.

Table 2. Major preclinical findings using FR-targeted nanoprobes for tumor imaging

Nanoprobe	Imaging technique	Model	Route	Major in vivo findings	Ref.
Self-assembly NPs of the heparin-FA conjugates and NIR fluorescence dye-780 (HF-IR-780 NPs)	NIR fluorescence imaging	MCF-7 tumor-bearing nude mice	i.v.	<ul style="list-style-type: none"> ● The fluorescence signal in the tumor was detected at 3 h post-injection, and the signal intensity was observed much stronger in tumor tissue than that in lung and liver at 72 h post-injection. ● HF-IR-780 NPs treated mice showed stronger fluorescence intensity in the tumor tissue than free IR-780 in the excised tissue evaluation at 72 h post-injection. 	[12]
FA-modified trypsin-stabilized gold nanoclusters with NIR fluorescence (FA-try-AuNCs)	NIR fluorescence imaging	Hela tumor-bearing nude mice	i.t. s.c.	<ul style="list-style-type: none"> ● The fluorescence signal was detected immediately after i.t. injection of FA-try-AuNCs, and the signal in the tumor can remain up to 12 h. ● The fluorescence signal was detected all over the whole body of the mice at 5 min post-s.c. injection of FA-try AuNCs, but the signal attenuation in the tumor site was slower than that in the normal tissues. 	[63]
FA-modified fluorenyl derivative-encapsulated silica NPs (SiNP-DFP-PEGFA)	NIR fluorescence imaging	HeLa tumor-bearing nude mice	i.v.	<ul style="list-style-type: none"> ● The fluorescence signal was detected in the tumor after 30 min post-injection of SiNP-DFP-PEGFA. ● The tumor fluorescence intensity was steadily increased and reached its maximum at 6 h post-injection. ● No significant fluorescence signal was detected in the tumor of the mice injected with SiNP-DFP-PEG 	[64]
Self-assembled NPs with poly- γ -glutamic acid-FA conjugates and fluorescently labeled chitosan conjugates with gadolinium complexed (PGA-FA/CH-A546-Gd NPs)	MRI	HeLa tumor-bearing nude mice	i.v.	<ul style="list-style-type: none"> ● The MRI signal intensity in the tumor site was increased 34% at 2 h post-injection of PGA-FA/CH-A546-Gd, compared to the untreated mice. 	[65]

FA-modified PEGylated poly(ethylene glycol)-poly(ϵ -caprolactone) micelles with SPIONs-encapsulated (FA-PEG-PCL-SPIONs)	MRI	BEL-7402 tumor-bearing nude mice	i.v.	<ul style="list-style-type: none"> ● The change of MRI signal intensity was increased by 2.5-fold in the tumor site at 3h post-injection of FA-PEG-PCL-SPIONs, compared to the one of PEG-PCL-SPIONs treated mice. ● An ideal post-injection time window for MRI scanning was within 6 h for FA-PEG-PCL-SPIONs. 	[66]
Radioiodinated tyrosine-click-FA conjugates (^{125}I -tyrosine-click-folate)	CT	KB tumor-bearing nude mice	i.v.	<ul style="list-style-type: none"> ● The CT signal in both tumor and kidney was achieved at 1 h post-injection of ^{125}I-tyrosine-click-folate. ● Pre-injection of potassium iodide and antifolate pemetrexed to the mice effectively reduced CT signal in the non-targeted organs (thyroid gland and kidney), improving tumor-to-background contrast. 	[67]
FA-modified dendrimer-entrapped gold NPs (Au DENPs-FA)	CT	SPC-A1 tumor-bearing nude mice	i.v. i.t. i.p.	<ul style="list-style-type: none"> ● Au DENPs-FA were uptaken by tumor tissue via i.v., i.t. or i.p. injection, allowing for effective CT imaging of tumor. ● Addition to the tumor, a large amount of Au DENPs-FA was found in kidney, lung, spleen and liver, allowing these NPs to be cleared through renal route and reticuloendothelial system. 	[68]
Gadolinium-loaded Au DENPs-FA (Gd-Au DENPs-FA)	CT/MR dual mode	KB tumor-bearing nude mice	i.v.	<ul style="list-style-type: none"> ● Both CT value and MR signal in the tumor site were increased by 200% and 152.7%, respectively at 24 h post-injection of Gd-Au DENPs-FA, compared to the ones of Gd-Au DENPs treated mice, allowing targeted dual mode CT/MR imaging of tumors. ● These NPs were cleared out from the body at 96 h post-injection. 	[69]

Abbreviations: NPs, nanoparticles; FA, folic acid; i.v., intravenous; i.t., intratumoral; i.p., intraperitoneal; s.c., subcutaneous; NIR, near-infrared; MR, magnetic resonance; CT, computed tomography.

7. Discussion and outlook

FA-decorated nanomedicines and nanoprobe have shown promising treatment outcomes for treating FR-positive tumors in mouse xenograft models. The potencies of these therapies *in vivo* are highly depended on 1) the pharmacokinetics (PK) and bioavailability of FA-decorated nanoparticles, 2) the binding affinity of FA-decorated nanoparticles to the FR on cancer cell surface, 3) the dose of FA-decorated nanoparticles that will saturate the FR on cancer cell surface, 4) the rate of folate receptor-mediated endocytosis of FA-decorated nanoparticles, 5) the release kinetics of drug or gene from the nanoparticle cargos, and 6) the recycling rate of FR back onto cancer cell surface for the next available FA-decorated nanoparticles [70].

One of the drawback of FR-targeted nanomedicines could be the high renal uptake. From the biodistribution evaluations, a large amount of the FA-decorated nanoparticles could be clearly observed in the liver and kidney [44, 67, 71]. Nanoparticles may accumulate in liver, spleen and lung because of their size distribution, i.e., nanoparticles with size of 50-100 nm tend to accumulate in the liver due to noncontinuous endothelia with vascular fenestrations; nanoparticles with the size of 200-500 nm tend to accumulate in spleen due to interendothelial cell slits; and microparticles with the size of 2-5 μm tend to accumulate in the capillaries of the lung [72]. Interestingly, nanoparticles only with the size less than 5 nm can be filtered out by the kidneys [73], which leads to the first logical question why these FA-decorated nanoparticles accumulated in the kidneys. Physiologically, folate binding protein (FBP), including FR α , FR β

and the reduced folate carrier (RFC), is highly expressed in the kidneys [74]. FBP that possesses high folate binding affinity is located in the brush board membrane of the proximal tubule cells to participate folate reabsorption from the convoluted proximal tubules [75-78]. Hence, these proximal tubular cells are not directly exposed to the circulated FA-decorated nanoparticles unless they are filtered from glomeruli. The filtration initially occurs at the interface of glomerulus and Bowman's capsule. Filtered molecules or nanoparticles need to pass through three filtration layers, the endothelium, glomerular basement membrane (GBM), and foot processes of the podocytes [79]. The endothelium provides an initial physical barrier that contains a pore size of 80-100 nm in diameter. The GBM with a high concentration of heparin sulfate in the middle layer provides negatively charge to electrostatically repel negatively charged molecules but attract positively charged molecules or nanoparticles, e.g., dendrimers, from the blood. The foot processes of podocytes with a pore size of 15-20 nm in diameter provide the last filtration barrier [80, 81].

By understanding the anatomy of the glomerulus and Bowman's capsule, the next question comes to what size, shape, zeta potential or other parameters allow FA-decorated nanoparticles to be filtered from the glomeruli and get reabsorbed or taken up by the proximal tubular epithelial cells. Recently, a folate-antioxidant conjugate (5-hydroxy-Tempo-folate) was synthesized to ameliorate renal ischemia/reperfusion injury (IRI). Such Tempo-folate conjugate was designed to target FR α -highly expressed proximal tubular epithelia, where the predominant site of injury occurred during and after IRI [82]. The third question is how to prevent FA-decorated

nanoparticles to be filtered from glomeruli to prolong the circulation time for systemic targeted chemotherapy, perhaps by increasing the size and PEGylation of the nanoparticles. Further investigations with more carefully designed experiments are needed to answer all these questions.

Chemotherapeutic agents, such as cisplatin, doxorubicin, methotrexate, etc., commonly cause nephrotoxicity, including any combination of glomerular or tubular dysfunction, hypertension and disturbance of the renal function [83, 84]. For example, cisplatin can cause acute kidney injury (AKI) and chronic kidney disease (CKD) by reducing renal perfusion, inducing morphologically necrosis of the terminal portion of the proximal tubule and apoptosis in the distal nephron, and eventually leading to renal failure [85-87]. Doxorubicin can cause Adriamycin (doxorubicin)-associated nephropathy (AAN) by inducing glomerulosclerosis and lead to the CKD [88-90]. Accumulation of FA-decorated nanoparticles containing chemotherapeutic agents in kidneys may increase the risk of impaired renal function. Indeed, it would be important to investigate the renal function, i.e., blood urea nitrogen (BUN), serum creatinine, and glomerular filtration rate (GFR), in addition to the current biocompatibility evaluation settings from these treated mice. Better understanding the biodistribution, toxicity, and renal clearance of FA-decorated nanoparticles/nanomedicines may help to guide FR-targeted strategies to advance anticancer chemotherapy in the (pre)clinical trials.

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